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Preclinical pharmacology of the anthrapyrazole analog oxantrazole (NSC-349174, Piroxantrone)

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Summary. Oxantrazole (now designated as piroxantrone) is an anthrapyrazole analog under evaluation as a potentially useful anthracycline-like antitumor agent. In preparation for phase I clinical trials, we characterized certain aspects of oxantrazole preclinical pharmacology, including plasma stability, murine pharmacokinetics, in vitro/in vivo metabolism, and DNA damage following incubation with human tumor cells in culture. Oxantrazole was relatively unstable in fresh mouse and dog plasma and particularly unstable in fresh human plasma ($t_{1/2}$ < 5 min at 37° C). Its decomposition in plasma was prevented by the addition of ascorbic acid, suggesting oxidative degradation. Following rapid i.v. administration of oxantrazole to mice, plasma elimination was best described by a two-compartment open model with an elimination-phase half-life, total body clearance, and steady-state volume of distribution of 330 min, 458 ml/min per m^2, and 87.9 l/m^2, respectively. The c × t value calculated following i.v. administration of 90 mg/m^2 oxantrazole to mice was 177 μg-min/ml. This value was subsequently used in a pharmacologically guided dose-escalation scheme for the oxantrazole phase I clinical trial. Oxantrazole was converted to a polar conjugate, presumably a β-glucuronide, by rat but not mouse hepatic microsomal preparations and in vivo by the mouse. Oxantrazole introduced protein-associated DNA strand breaks following incubation with a human rhabdomyosarcoma cell line. Repair of the damage was complete by 15 h. Clinical pharmacologic studies are currently under way in conjunction with the phase I clinical trial of oxantrazole.

Introduction

The anthrapyrazole derivative oxantrazole (NSC-349174, Fig. 1) is an anthracenedione congener currently under evaluation as an antitumor agent. Oxantrazole is one of many modified anthracenediones synthesized by Warner-Lambert/Parke-Davis in an effort to decrease the cardiotoxicity associated with anthracycline-like compounds by reducing the potential for semiquinone free-radical production often associated with these molecules [16, 17]. Superoxide dismutase-sensitive oxygen consumption in rat liver microsomal preparations was reduced for oxantrazole as compared with adriamycin [6], and oxantrazole was less toxic than adriamycin in a fetal mouse heart organ-culture cardiotoxicity model system [8]. Additional biochemical studies showed that oxantrazole intercalated into DNA, inhibited DNA synthesis in L1210 cells, and introduced DNA strand breaks when incubated with L1210 cells in culture [6].

Oxantrazole showed activity against model tumor systems, including L1210 leukemia, B16 melanoma, M5076 sarcoma, and the MX-1 mammary xenograft in nude mice [10, 14], but had no activity against several anthracycline-resistant tumor cell lines [10, 14]. No marked schedule dependency was observed with i.p. implanted L1210 leukemia; however, survival was slightly reduced on the daily ×9 as compared with the 1-day schedule [14]. Modest activity was observed following i.v. but not oral administration. Preclinical toxicology studies in mice and beagle dogs have recently been completed [11]. The single-dose LD_{10}, LD_{50}, and LD_{90} in mice were 75 mg/m^2 (25 mg/kg), 99 mg/m^2 (33 mg/kg), and 132 mg/m^2 (44 mg/kg), respectively [11]. The mouse equivalent LD_{10} ($MELD_{10}$) and 1/10 $MELD_{10}$ for the beagle dog were 76.2 mg/m^2 (3.81 mg/kg) and 7.6 mg/m^2 (0.38 mg/kg), respectively. Following administration of the $MELD_{10}$ dose to dogs, reversible myelosuppression and testicular atrophy were observed, but no renal or hepatic toxicities were noted at that dose. Much less severe myelosuppression and testicular atrophy were observed at 1/10 the $MELD_{10}$ dose in dogs.

In preparation for subsequent preclinical and clinical pharmacologic investigations, we developed a reversed-phase HPLC assay for oxantrazole in biological fluids [5]. We report the results of preclinical pharmacology, pharmacokinetic, and metabolism studies with oxantrazole. Included are c × t data obtained following rapid i.v. infusion of oxantrazole to mice. These data are important in the pharmacologically guided dose-escalation scheme [3] currently used in oxantrazole phase I clinical trials.

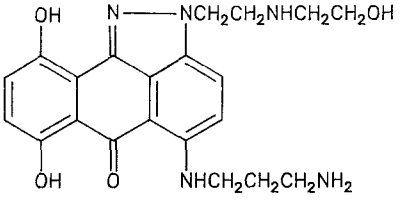

Fig. 1. Structure of oxantrazole (NSC-349174)

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Materials and methods

Materials. Oxantrazole was supplied as the dihydrochloride salt and formulated product by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md). The internal standard (PD 111816) was generously provided by Warner-Lambert/Parke-Davis (Ann Arbor, Mich). Disposable extraction columns (C_{18}, 1 ml) were purchased from J. T. Baker (Phillipsburg, NJ). Hibar RP-2 10-μ HPLC columns were obtained from E. M. Merck (Darmstadt, West Germany). Methyl-[^{3}H]- and methyl-[^{14}C]-labeled thymidine were purchased from Dupont New England Nuclear (Wilmington, Del). 3-Methylcholanthrene was purchased from Pfaltz and Bauer (Stanford, Conn). Fetal calf serum, trypsin, and Dulbecco's modified Eagle medium (DMEM) were obtained from Gibco (Grand Island, NY). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim (Indianapolis, Ind). Resorcinol, phenothalein glucuronide, adriamycin, EDTA, and uridine diphosphoglucuronic acid (UDPGA) were obtained from Sigma (St. Louis, Mo). All other reagents and solvents were of reagent or chromatographic grade. Male CD_2F_1 mice were supplied by Charles River Farms (Lafayette, Ind). Male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, Ind). A204 human rhabdomyosarcoma cells were obtained from American Tissue Type Collection (Rockville, Md).

HPLC analysis of oxantrazole. The reversed-phase HPLC assay for oxantrazole in biological fluids has previously been described [5]. The limit of detection for the assay was 10 ng/ml plasma. Briefly, plasma and urine were diluted by the addition of 0.05 *M* sodium phosphate buffer (pH 6.0), and to that mixture was added 25 μl 1.2 *M* citric acid, 25% sodium ascorbate (pH 4) solution, and internal standard. The drug and internal standard were isolated from plasma by elution from disposable C_{18} extraction columns with methanol/glacial acetic acid/0.02 *M* ammonium acetate buffer, pH 4.0 (12/1/3). Samples were analyzed by reversed-phase chromatography with a 5-cm pellicular C_3 guard column, a C_2 analytical column, and a mobile solvent system of dimethylformamide/acetonitrile/0.2 *M* sodium acetate (pH 4.5) (20/5/75). The drug, internal standard, and metabolites were detected by UV absorbance at 514 nm. Plasma and urinary concentrations were determined by fitting unknown sample/internal standard ratios to the linear regression equations of standard curves.

Plasma stability studies. Oxantrazole was incubated for 24 h in thawed and fresh human (Mayo Clinic Blood Bank), fresh mouse, and fresh dog plasma obtained by centrifugation of citrated whole blood. Following the addition of 50 μl 1.2 *M* citric acid, 25% sodium ascorbate solution, and internal standard to plasma aliquots (50–100 μl), samples were rapidly frozen (dry ice/methanol) and stored at −20° C.

Mouse pharmacokinetic studies. Male CD_2F_1 mice were housed on commercial wood shaving bedding and allowed Purina Mouse Chow and tap water ad libitum. Stock solutions (20 mg/ml) of oxantrazole were prepared by adding sterile water to formulated drug and were diluted (saline) such that a 20-g mouse received a 120-μl injection for a dose of 90 mg/m^2. Mice were placed in a Broome tail cuff restraint and the drug was injected over 30 s into a lateral tail vein with a 27-gauge needle attached to a tuberculin syringe. Blood was collected by cardiac puncture and plasma immediately obtained by rapid centrifugation of the citrated whole blood. In all, 50 μl 1.2 *M* citric acid, 25% sodium ascorbate (pH 4) solution was immediately added to plasma samples, and samples were frozen at −20° C until analysis. Pharmacokinetic data were analyzed using the NONLIN least-squares regression analysis program [13] on a CDC Cyber 170-720 computer equipped with interactive graphic analysis. The biexponential decline in oxantrazole plasma concentration was fitted to the equation $C = Ae^{-\alpha t} + Be^{-\beta t}$ with a weighting factor of $1/C$, where C is the plasma concentration of oxantrazole at time t following administration of the oxantrazole, A and B are the intercepts at $t = 0$, and α and β are the fast and slow disposition rate constants, respectively.

Metabolism studies. Male CD_2F_1 mice (25–35 g) and male Sprague-Dawley rats (200–300 g) were used for metabolism studies. For enzyme induction studies, animals were pretreated with 3-methylcholanthrene on day 1 (40 mg/kg dissolved in olive oil) and day 3 (20 mg/kg dissolved in olive oil) and sacrificed on day 5. Hepatic microsomes were prepared by differential centrifugation of liver homogenates [4]. For oxidative metabolism studies, microsomal incubations (37° C) contained microsomal protein (3 mg/ml), TRIS buffer (50 m*M*, pH 7.4), NADP+ (.42 m*M*), glucose-6-phosphate (24 m*M*), glucose-6 -phosphate dehydrogenase (0.7 IU/ml), magnesium chloride (5.0 m*M*), and oxantrazole (0.5 m*M*) in a final volume of 3 ml. For glucuronidation studies, microsomal incubations (37° C) consisted of microsomal protein (3 mg/ml), uridine diphosphoglucuronic acid (UDPGA) (4 m*M*), TRIS buffer (50 m*M*, pH 7.4), and oxantrazole (0.3 m*M*) in a final volume of 6 ml. Aliquots (0.1 ml) were removed from incubation mixtures and added to equal volumes of ice-cold acetonitrile. Following centrifugation, the supernatant was diluted with mobile solvent and analyzed by HPLC. Alternatively, microsomal incubation aliquots were applied to C_{18} disposable extraction columns and parent drug and metabolites were eluted as for plasma samples.

The presence of glucuronide metabolites in urine and in UDPGA-fortified microsomal incubations was evaluated by two methods. Urine and the microsomal metabolite (purified by HPLC from UDPGA-fortified microsomal incubations) were incubated with sulfatase-free β-glucuronidase in 50 m*M* sodium phosphate (pH 6.8) for 24 h at 37° C. The disappearance of metabolite and appearance of parent drug were assessed by HPLC. Control samples were incubated in the absence of enzyme. The presence of glucuronides was also assessed using the naphthoresorcinol colorimetric assay [12]. Briefly, conjugated D-glucuronic acids were determined by heating samples in base followed by reaction with the naphthoresorcinol reagent. Absorbance at 564 nm for urine and purified microsomal metabolite was compared to control mouse urine samples and control solvent samples, respectively. The positive control was phenothalein glucuronic acid.

Alkaline elution. DNA damage was evaluated by the method of Kohn et al. [9], with minor modifications. For alkaline elution studies, 1×10^5 human rhabdomyosarcoma

(A204) cells were plated in 25-cm^2 flasks and DNA was labeled for 24 h with 0.02 μCi/ml methyl-[^{14}C]-thymidine (0.36 nmol/ml). The labeling period was followed by a 24-h incubation in fresh media prior to alkaline elution. L1210 cells (2.5×10^5 cells/ml) were labeled with 0.01 μCi/ml methyl-[^{3}H]-thymidine (0.91 nmol/ml) and used as internal standard cells 24 h later. Stock solutions of oxantrazole were prepared by dissolving drugs in 0.1 *N* HCl and final dilutions (distilled water) were made just prior to the addition of oxantrazole to A204 cells. Following a 1-h exposure (37° C), drug-containing media was poured off, cells were rinsed with fresh media, and additional media were added to cells. For DNA repair studies, A204 cells were exposed to drug for 1 h and then incubated in fresh media (37° C) for various periods of time prior to alkaline elution analysis. Following drug exposure and repair, approximately 5×10^5 A204 experimental cells were briefly treated with trypsin, irradiated (where appropriate), and added to 50-ml tubes containing 20 ml cold, 5 m*M* phosphate-buffered saline (pH 7.4). L1210 internal standard cells (5×10^5 cells) were irradiated (150 R) and added to each tube. Cells were loaded slowly onto polyvinyl chloride filters and phosphate buffer was removed by mild suction. The lysis solution (3 ml) of 2% SDS and 0.02 *M* EDTA (pH 10) was added to funnels and allowed to flow by gravity. When cell extracts were treated with proteinase K, 1 ml 0.5 mg proteinase K/ml lysis solution was added to the funnel and allowed to stand for 15 min. The alkaline elution buffer (0.02 *M* EDTA and tetrapropylammonium hydroxide to pH 12.1) was added to funnels and DNA was eluted at a rate of 0.02 ml/min, with fractions collected every 3 h for 15 h. Radioactivity in fractions, remaining on filters, and in the lines was determined as previously described by Kohn et al. [9].

Results

Based on the rapid oxidation of oxantrazole in neutral and alkaline aqueous solutions [5], we characterized drug stability in plasma prior to pharmacology studies. Fresh human plasma degraded oxantrazole in a temperature-dependent manner much more rapidly than did fresh dog and mouse plasma (Table 1). The drug was degraded more slowly by an ultrafiltrate of fresh human plasma than by the unfiltered plasma (Table 1), although the addition of 1.2 *M* citric acid/25% sodium ascorbate to fresh human plasma greatly retarded the rate of drug degradation (Table 1). Accordingly, for mouse pharmacokinetic studies, rapid centrifugation of blood samples was followed by the addition of ascorbate/citrate to plasma to prevent drug decomposition.

Table 1. Stability of oxantrazole (25 μg/ml) in human, mouse and dog plasma

Plasma sample	Incubation temperature	Oxantrazole $t_{1/2}$ (h)
Fresh human	4° C	3.19
	25° C	0.21
	37° C	0.08
Fresh dog	37° C	0.63
Fresh mouse	37° C	1.75
Human ultrafiltrate	25° C	4.33
Fresh human with citrate/ascorbate[a]	25° C	>10

[a] 50 μl 1.2 *M* citric acid/25% sodium ascorbate (pH 4.0)/ml plasma

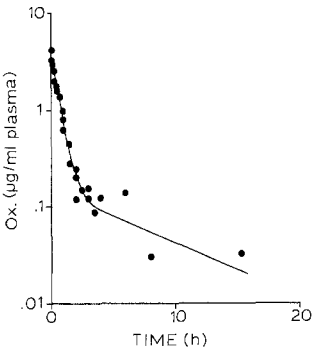

Fig. 2. Plasma concentration-time curve following rapid i.v. administration of oxantrazole (90 mg/m^2) to mice. Each symbol represents one animal, and the data were collected from three experiments

Plasma elimination of oxantrazole was characterized following i.v. infusion of 90 mg/m^2 to mice. Plasma concentration-time data for the study are illustrated in Fig. 2. Oxantrazole plasma elimination was best described by a two-compartment open model; pharmacokinetic parameters are summarized in Table 2. In mouse urine collected for 24 h following exposure, <5% of the oxantrazole dose was recovered as the parent drug. In preparation for NCI-sponsored phase I clinical trials of oxantrazole, the c × t value for the data illustrated in Fig. 2 was calculated in collaboration with Dr. Jerry Collins at NCI. The c × t value was 177 μg-min/ml for the 90-mg/m^2 dose. The relevant, target mouse c × t value based on the pharmacologically guided dose-escalation scheme [3] used in the phase I trial was 40% of the mouse LD_{10} (75 mg/m^2) c × t value. After adjusting for the LD_{10} dose factor (75/90), the 40% target value calculated was 59 μg-min/ml.

In addition to the parent drug, urine from oxantrazole-treated mice contained a metabolite that eluted more rapidly (3.1 min) than the parent drug (5.5 min). The UV spectrum of the metabolite was very similar to that of the parent drug, with absorptions at 500–560, 490, and 400 nm (data not shown). Subsequently, oxantrazole was incubated with mouse-liver microsomal preparations containing either an enzymatic NADPH-generating system or UDPGA to evaluate its oxidation and glucuronidation, respectively. No disappearance of the parent drug or appearance of metabolites was observed following incubation of oxantrazole with preparations from control or 3-methylcholanthrene-treated animals. Similarly, when oxantrazole was incubated with preparations from control or 3-methylcholanthrene-treated rats and an enzymatic NADPH-generat-

Table 2. Pharmacokinetic parameters for rapid i.v. infusion of oxantrazole (90 mg/m^2) to male mice

$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (min)	Cl_{TB} (ml/min per m^2)	(ml/min per kg)	Vd_{ss} (l/m^2)	(l/kg)
22	330	458	151	87.9	28.9

ing system, no evidence of metabolism was noted. However, when it was incubated under identical conditions in the presence of rat (rather than mouse)-liver microsomal preparations and UDPGA, one metabolite appeared over time while oxantrazole disappeared in a time-dependent fashion. This rat microsomal metabolite had an HPLC retention time (3.1 min) and UV spectrum identical to that of the urinary oxantrazole metabolite of the polar mouse.

The presence of glucuronide metabolite(s) in the UDPGA-fortified rat microsomal incubations and in mouse urine was further studied in two ways. When the purified rat microsomal UDPGA-dependent metabolite was incubated for 24 h with sulfatase-free β-glucuronidase, there was time-dependent disappearance of the metabolite and appearance of the parent drug in HPLC chromatograms (Fig. 3). No changes were observed when β-glucuronidase was omitted from incubations. Similarly, incubation of mouse urine with β-glucuronidase resulted in decreased metabolite and increased parent-drug peak areas in HPLC chromatograms. The formation of a conjugated glucuronide by rat UDPGA-fortified microsomal incubations was also consistent with results of a colorimetric assay in which naphthoresorcinol reagent reacted with conjugated D-glucuronides [12]. When the HPLC-purified conjugate was allowed to react with the reagent, a positive result was obtained, based on absorbance at 564 nm compared with control samples or with samples containing oxantrazole rather than the metabolite. For a particular sample of purified metabolite, the metabolite concentration as determined by the naphthoresorcinol assay was 28 μ*M* (based on phenolthalein glucuronide as the standard), in close agreement with the value obtained by HPLC analysis based on parent-drug equivalents (22 μM). Increased absorbance was observed for urine samples from drug-treated mice as compared with those from control mice (data not shown).

The DNA-damaging effects of oxantrazole were evaluated using human rhabdomyosarcoma (A204) tumor cells in culture. Minimal DNA damage was observed following treatment of A204 cells with the drug at concentrations as high as 1.0 μ*M* (data not shown). In marked contrast, the

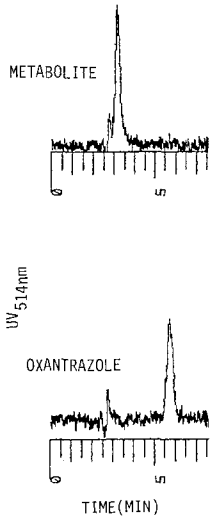

Fig. 3. Chromatograms of HPLC-purified metabolite from UDPGA-fortified microsomal incubations subsequently incubated with sulfatase-free β-glucuronidase. Zero time incubation is shown in *upper panel* and 24-h incubation, in *lower panel*

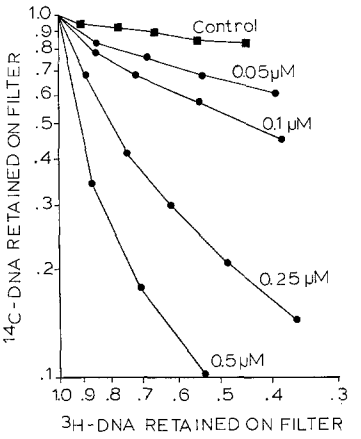

Fig. 4. DNA alkaline elution profile for A204 cells following 1-h incubation with oxantrazole. Cell lysates were treated with proteinase K

rate of DNA elution increased as a function of oxantrazole concentration (0.05–0.5 μ*M*) when cell lysates were treated with proteinase K prior to alkaline elution (Fig. 4), suggesting the formation of protein-associated DNA strand breaks. Protein-DNA cross-links retard the rate of elution of DNA from drug-treated and irradiated cells as compared with control irradiated cells. Elution rates for DNA from A204 cells treated with oxantrazole and irradiated were retarded compared with those from irradiated cells that were not incubated with the drug (data not shown). DNA strand breaks introduced by oxantrazole were no longer detectable 15 h after removal of the drug from cell incubations.

Discussion

Oxantrazole is one of several anthrapyrazole analogs currently under evaluation in the United States and Europe in an effort to develop anthracycline-like agents with reduced cardiotoxicity. In conjunction with NCI-sponsored pharmacologic and toxicologic evaluation of this new agent and as a prelude to phase I clinical evaluation at this institution, we conducted preclinical pharmacologic studies with oxantrazole. The proper evaluation and interpretation of pharmacologic data arising from oxantrazole studies required an appreciation of the instability of the parent drug in neutral and alkaline aqueous solutions [5] and plasma. Degradation was extensive and most likely involved oxidation, since ascorbic acid and acidic conditions prevented decomposition. The rapid degradation of oxantrazole in plasma as compared with plasma ultrafiltrate suggested that plasma protein substituents accelerated the rate of decomposition. Although rapid cooling and centrifugation of blood samples followed by the addition of ascorbate/citrate was adequate for preventing drug decomposition in mouse plasma, the more rapid oxidation in fresh human plasma required the collection of blood samples in citrate/ascorbate to assure drug stability (M. M. Ames, unpublished observations).

The pharmacokinetics of oxantrazole in mice (relatively prolonged plasma elimination, large volume of distribution, rapid clearance, and low urinary recovery of the parent drug) are similar to those of anthracyclines such as adriamycin and the anthracenedione mitoxantrone in mice

and humans. The rapid initial decline of plasma concentrations and prolonged terminal phases of plasma elimination of adriamycin in rats [19] and man [7, 19] have been assumed to be associated with rapid tissue accumulation and slow release of the drug. Large volumes of distribution and rapid body clearance such as those observed for oxantrazole in mice are the norm for anthracycline-like agents and have been reported for agents such as adriamycin [7, 19] and mitoxantrone [1, 18]. The rapid oxidation of oxantrazole in plasma may contribute to the plasma elimination of this agent.

Mouse pharmacokinetic data obtained in this study are being used in a novel manner for the phase I clinical trial of oxantrazole at this institution. Collins et al. [3] recently proposed two schemes for pharmacologically guided dose escalations in phase I trials as a means to use patient resources more efficiently while maintaining the safety features of current trial design. The rationale for such schemes was that new agents frequently require more than the predicted number of dose escalations in phase I trials, and the reduced toxicity in humans is often due to species differences in drug elimination and thus drug exposure. The approach requires determination of patient drug exposure ($c \times t$ values) during the phase I trial for comparison with mouse $c \times t$ data obtained from preclinical pharmacologic values. Escalations proceed at an optimal rate until patient $c \times t$ values reach a target value associated with a nontoxic dose in the mouse. The particular scheme incorporated into our oxantrazole phase I trial specifies dose escalations from the initial 1/10 mouse LD_{10} dose by factors of 2 (rather than by the modified Fibonacci method) until the human $c \times t$ value $\geqslant 40\%$ of the mouse LD_{10} $c \times t$ value [3]. We calculated a mouse $c \times t$ value of 177 μg-min/ml for a dose of 90 mg/m^2. After reducing the mouse $c \times t$ value by a factor of 75/90 based on the final mouse single-dose LD_{10} dose of 75 mg/m^2, the target $c \times t$ value of 59 μg-min/ml was not exceeded at the first three dose escalations (7.5, 15.0, 30, or 45 mg/m^2) in the phase I clinical trial [2].

In vitro metabolic studies with rat and mouse NADPH-fortified liver microsomes did not reveal oxidative metabolism. Rat but not mouse microsomes fortified with UDPGA converted oxantrazole to a polar metabolite identical in retention time and UV spectrum to a mouse urinary metabolite we believe to be a glucuronide, a conclusion supported by the β-glucuronidase hydrolysis of the metabolite as well as positive results in the naphthoresorcinol colorimetric assay. Rat microsomes have been reported to convert the structurally similar agent mitoxantrone to a glucuronide [20]. In these studies, neither of two polar human urinary metabolites of mitoxantrone were hydrolyzed by β-glucuronidase [20], whereas the rat microsomal metabolite was converted to the parent drug in the presence of the enzyme [18]. We were surprised that mouse microsomal preparations did not catalyze the glucuronidation of oxantrazole. It may be that the mouse liver is inefficient in the formation of such glucuronides, but the urinary data (β-glucuronidase hydrolysis, HPLC retention time, UV spectrum) are consistent with the presence of the glucuronide following i.v. administration of oxantrazole to the mouse.

Fry et al. [6] previously reported that oxantrazole is a DNA strand-breaking agent in L1210 cells, as are many anthracyclines and related molecules. We obtained similar results with human rhabdomyosarcoma cells. DNA strand breaks were protein-associated, as is true for agents such as adriamycin, ellipticine, and VP-16 [15]. These findings are consistent with the possibility that oxantrazole interacts with topoisomerase II, leading to enzyme-DNA-drug lesions detected as single strand breaks, but only in the presence of proteinase K.

The phase I clinical trial and pharmacology study are currently underway. The pharmacokinetic and metabolic data reported in the present study will be of interest in characterizing the behavior of this agent in patients.

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